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Research Article:

Inflammatory and Immunoregulatory Cytokine Responses to Genital Mycoplasma and Other Vaginal Pathogens among Women in Anambra State, Nigeria

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ABSTRACT:
Background: Genital mycoplasmas and other vaginal pathogens may influence host inflammatory responses, but their relationship with cytokine profiles remains incompletely understood. This study assessed the levels of interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) among patients with genital mycoplasmas and other vaginal pathogens. Methods: A comparative cross-sectional approach was used to evaluate IL-1β and IL-10 concentrations among participants who tested positive or negative for Mycoplasma, Ureaplasma, and selected vaginal pathogens. Cytokine concentrations were compared between pathogen-positive and pathogen-negative groups using analysis of variance, with statistical significance set at $p < 0.05$. Results: IL-1β concentrations did not differ significantly between positive and negative groups for any of the pathogens investigated. For Mycoplasma genitalium, mean IL-1β concentrations were 16.36 ± 1.92 and 16.16 ± 2.99, respectively ($p = 0.813$). Similarly, no significant differences were observed for M. hominis, Ureaplasma urealyticum, U. parvum, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Candida species, haemolytic streptococci, or Trichomonas vaginalis. IL-10 levels tended to be higher among participants positive for M. genitalium (526.13 ± 172.21 vs. 426.52 ± 271.50) and U. parvum (521.11 ± 167.76 vs. 429.09 ± 270.06), although these differences were not statistically significant ($p = 0.192$ and 0.371, respectively). Conclusion: Genital mycoplasmas and other vaginal pathogens were not significantly associated with systemic IL-1β or IL-10 concentrations. The observed tendency toward increased IL-10 among some pathogen-positive groups may reflect an immunoregulatory response and warrants further investigation using larger, longitudinal studies and locally measured genital cytokine profiles.
Keyword: IL-1β, IL-10, genital mycoplasma, Ureaplasma, vaginal pathogens.

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INTRODUCTION

Mycoplasma belongs to the family of Mycoplasmataceae in the class of bacteria called Mollicutes. They are the smallest bacteria that can grow on cell-free medium. Their characteristic cell wall deficient nature made them highly pleomorphic in shape. While conferring on them resistance to beta lactam and other antimicrobial agents that act on cell wall of bacteria and prevent them from being stained by the Gram's reagents. Sexually transmitted infection (STI) is still a burden for Nigerian women because it is asymptomatic. The lack of detection of genital mycoplasmas in apparently healthy sexually active females in Nigeria is due to non-application of high throughput molecular approach [1]. Genital Mycoplasmas are commensals that can be detected in the lower genito urinary tract of sexually active women, resulting in colonization of these genitalia by sexual contact. Vaginal colonization of this bacteremia mainly causes vaginosis, postpartum fever, pelvic inflammatory disease, infertility, and postpartum

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septicemia, and preterm labor, premature rupture of the membranes, systemic neonatal infections and preterm birth [2]. Human genital Mycoplasma may be transmitted by direct contact between host through genital-genital contact or oral-genital contact, it can also be transmitted vertically from mother to offspring either at birth or by nosocomial acquisition through transplanted tissue [3]. Colonization of these organisms after puberty has been reported to occur primarily as a result of sexual contact, since sexually matured persons with no history of sexual contact are infrequently colonized with genital mycoplasmas [4]. It has also been observed that colonization increased more rapidly with increasing sexual experience in women than in men, thus suggesting that women are more susceptible to colonization with these organisms [5].

There are increasing cases of sexually transmitted diseases due to social activities and Human Immunodeficiency Virus is among sexually transmitted disease with great derangement to immunity leading to opportunities for increased deaths. Cross-sectional studies have shown that a strong association between genital Mycoplasma infection and HIV infection [6]. Studies among HIV-positive women have revealed prevalence rates of genital Mycoplasma as 7.1% and 3.0% [7-8]. The role of genital Mycoplasmas as disease causing agent has been reported in many parts of the world. Although very limited studies have been done in Nigeria none has been carried out on HIV patients. Despite reports on these organisms, they are still not being investigated routinely mainly due to their fastidious nature and the technically challenging culture methods needed to link the organisms to clinical conditions. Infection with Human Immunodeficiency Virus (HIV) is characterized by complex pathological changes in which the immunological system shows a significant reduction in T-helper cell-dependent cellular immune response. Human Immunodeficiency Virus is recognized as the causative agent of acquired immune deficiency syndrome (AIDS). However, the progression of AIDS is highly variable in different individuals and factors, such as viral strains or most factors, has been associated as the possible cause of variations [9]. Infectious agents including various viruses, parasites and bacteria are considered to be the co-factors in the progression of AIDS [10]. In Nigeri*a* as well as other developing countries, little is known about the prevalence of genital Mycoplasmas among HIV patients, therefore the study is aimed to screen patients for the bacteria, determine the level of cytokines in patient's serum and subsequently search for the presence of virulent genes from the positive samples of patients attending the HIV clinic of Nnamdi Azikiwe Teaching Hospital Nnewi, South Eastern, Nigeria.

MATERIALS AND METHODS

Study Design

The research was a cross-sectional analytical study involving 256 randomly selected female patients who have tested positive to HIV infection, aged 18-60 years and who consented to the study. High vaginal swab samples were collected from the subjects and were analyzed microbiologically. Genomic DNA was extracted from each specimen and subjected to PCR analysis. Semi-structured, pre-tested questionnaires were used to collect relevant bio-data from the participants. The results were analyzed using Chi-square and percentages.

Study Site

The study sites were Nnamdi Azikiwe University Teaching Hospital, Heart to Heart Centre Nnewi, Anambra State, Nigeria. Geographically, Nnewi with a total population of 391,227 falls within the tropical rain forest region of Southeastern Nigeria, and Coordinates in latitude 6 ^{0}1'0"N and longitude 6 ^{0} 55'0"E. The hospital is a tertiary health facility and referral centre accessed by patient's resident in Nnewi and its environs. It was officially commissioned on July of 1991 but the permanent site, located at Akammili, Nnewi was inaugurated in May, 2023. The hospital Consists of five out posts located at Ukpo, Oba, Awka, Neni and Umunya. Presently, the Staff Strength of the hospital has grown to approximately 3000 Medical and non-medical Staff as at 2023.

SAMPLE SIZE DETERMINATION

The sample size for the study was 256 apparently healthy HIV positive female patients.
This was derived from the formula below
$N = Z^2(1-P) / d^2$ [11]
Where
N= Sample Size
Z score = 1.96 at 95% confidence interval
P = expected prevalence rate
D =Desired level of significant 0.05 (5%)

Substituting
Z =1.96 for level of confidence of 95%
P =20%(0.20) prevalence of Genital Mycoplasma in Nnewi, Anambra State, Nigeria. [12]
D = 5% (0.05)
$N=z^2p(1-p)/d^2=1.96^2x0.20(1-0.20)/0.05^{2=}$ 245.86

Study Population

Two hundred and fifty female HIV positive patients of the Nnamdi Azikiwe University Teaching Hospital Nnewi aged between 18 and 60 years were enrolled into the study. Informed consent was obtained from the subjects before been recruited into the study.

The Sampling technique employed was simple random sampling.

Ethical Considerations

Informed consent of each participant was obtained prior to sample collection and testing. Ethical approval was also obtained from Research Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi.

Inclusion and Exclusion Criteria

All Subjects who gave informed consent were included in the study. Subjects who declined to offer consent was excluded from the study.

The Inclusion Criteria for the subjects was as follows:

- Age range of 18 to 60 years
- Sexually active female's HIV patients
- Female HIV positive patients not on antibiotics therapy within the last three months
- The exclusion criteria for all Subjects included:
- Female HIV positive patients on antibiotic and antifungal medication a month before specimen collection
- Female HIV positive patients who declined informed consent
- Female HIV patients on menstrual period at the time of the study.

Specimen Collection

Prior to collection of samples from the female subjects, series of health talks were conducted for the participants after the Head of the Department and ethics Committee had given their approval for the study. Blood Specimens were collected by venepuncture from all individuals enrolled in the study for the following assay.

1 HIV screening
2 CD4 Count
3 Inflammatory cytokine (IL -10 and IL-1β)

Blood Specimen was collected and approximately separated as whole blood, serum or plasma and preserved accordingly by standard method before testing was performed.

HIV Screening

A Determine HIV-1/2 kit and Uni-gold HIV was used for first line and second line HIV screening.

Procedure

As described by the manufacturer, 50µl of patient samples were separated from the clotted whole samples and were delivered onto appropriately labelled specimen pads. After minutes of application, the result was read. This method has in-built quality control and double line on the test region represents a HIV sero-positive reaction while a single red line in the test region labelled control only represents an HIV sero -negative reaction.

Uni-Gold HIV KIt

Procedure

The procedure followed was described by the manufacturer. Two drops of blood sample were dropped on the sample port, followed by two drops of wash solution and allowed to react for ten minutes. This method also has inbuilt quality control. The presence of two pink/purple line at the control region indicates a HIV sero-negative reaction.

Culture

Media Preparation

Oxoid products (Mycoplasma agar base CMO 401) Mycoplasma broth base (CM 0401) Mycoplasma. Supplement G (SR 0059) and Mycoplasma Supplement P (SR 0060) were reconstituted according to manufactures instruction. HVS was inoculated into Oxoid Mycoplasma broth medium and Oxoid Ureaplasma broth medium in Bijou bottles after collection on site. Inoculated bottles were subsequently delivered to the Medical Microbiology laboratory for inoculation and isolation of the organism. All inoculated broth was incubated aerobically for 48 hours for Ureaplasma and up to 7 days for Mycoplasma and were examined periodically for colour changes for Mycoplasma, there will be production for colour change of phenol red indicator to pale yellow. Broth cultures for Ureaplasma spp. were examined for color change resulting from hydrolysis of Urea twice daily for up to 7 days because of the rapid decline in viability of these organisms in culture.

Sub-culturing on Mycoplasma and Ureaplasma Agar medium.

Bottles showing color change were sub cultured unto agar plates (usually 55mm) that were prepared with Mycoplasma Agar Base CM0401, Mycoplasma supplement–G SR 0059, L-arginie and Urea for Ureaplasma for further examination. The culture plates were incubated into a candle jar for increase Carbondioxide atmosphere. Incubation was for 24-48 hours for Ureaplasma media and up to 10 days for Mycoplasma media. Suspected colonies were examined with a dissecting microscope for "fried –egg" appearance.

Biochemical Test for Confirmation of Isolates

1 Carbohydrate fermentation by Mycoplasma was tested by incorporating 1% W/V carbohydrate (L-Arginine and glucose) and 0.005% W/V phenol red into the broth medium. *Mycoplasma hominis* and *Mycoplasma genitalium* will digest the arginine and glucose leading to production of acid resulting in a colour change of the phenol red indicator to yellow.
2 Urea production by Ureaplasma
3 Hydrolysis of Urea by Ureaplasma was tested by incorporation of 1% W/V Urea and $CaCl_2$

indicator. Urea hydrolysis in broth cause an alkaline shift from orange to deep red.

Isolation of Routine Vaginal Pathogen
Specimen: The third Vaginal Swab was used.

Media: Blood agar, Chocolate, MacConkey, and Saboursaud dextrose agars were prepared according to the manufacturer s instruction

Culture Method: Each Swab was inoculated on the above media and incubated aerobically for 24 hrs at 37ºC after which the isolates were identified using Bacteriologic methods.

Gram's reaction: After inoculation into the above media, the swabs were used to make a smear on a clean grease free slide and stained, by the Gram technique (Appendix xiv). The Gram stained smears were examined for the presence of Clue cells, yeast Cells and bacteria cells.

Wet Preparations: The Swab was expressed into two drops of physiological Saline. The sediments were examined under a light microscopy (40x magnification). The presence of *Trichomonas vaginalis*, yeast cells, Clue cells were also noted.

Cytokine evaluation of interleukin 1 β eta and interleukin 10 (IL_1β and IL_10)
Inter Leukin 1 Beta (Il-1β) Assay

I L – 1β Quantitative test is base on a solid phase enzyme linked immune assay (ELISA). The assay utilizes mouse monoclonal anti I L -1β anti body in the antibody enzyme (horseradish peroxidase) conjugate solution. Human interleukin 1β commercial Eliza kit by MELSIN MEDICAL COMPANY limited was used.in this method. IL-1β calibrators 50µl were added to the standard well, blank well was set with standard and sample dilution at 40µl for testing of sample well. Ten (10) µl of testing samples was added to make a final dilution of 5 folds A 100 µl volume of HRP-Conjugate reagent was added to each well, except the blank well. The plates were closed with closure plate membrane and incubated for 60mins at 37^0C.Washing was done 20 folds, diluted with distilled water and reserved. The closure plate membrane was uncovered, dried by swinging and the liquid discard. Wash buffer was added to every well for 30 secs and then drain five (5) times. A 50µl volume of chromogen solution A and chromogen B solution was added to each well and incubated for 15 mins at 37^0C after which a fifty (50) µl volume stop solution was added to each well which changes from blue colour to yellow colour. The blank well was set to zero and the absorbance read at 450nm within 15 mins using a micro plate reader to obtain values. A reference chart was used to calculate the concentration by comparing the opticl density (OD) of the samples to the standard curves respectively.

Interleukin 1 (I L – I0) Assay

I L – I0 Quantitative test is based on a solid phase enzyme linked immuno assay (ELISA). Human interleukin IL-IB commercial Eliza kit by Melsin Medical company limited was used. In this method, IL-I0 calibrators 50µl were added to the standard well, blank wellwas set with standard and sample dilution at 40 µl for testing of sample well. Ten (10) µl of testing samples was added to make a final dilution of 5 folds. A 100 µl volume of HRP-Conjugate reagent was added to each well, except the blank well. The plates were closed with closure plate membrane and incubated for 60 mins at 37^0C.Washing was done 20 folds, diluted with distilled water and reserved. The closure plate membrane was uncovered, dried by swinging and the liquid discared. Wash buffer was added to every well for 30secs and then drained five (5) times. A 50 µl volume of chromogen solution A and Chromogen B solution was added to each well and incubated for 15mins at 37^0C after which a fifty (50) µl volume stop solution was added to each well which changes from blue colour to yellow colour. The blank well was set to zero and the absorbance read at 450nm within 15mins using a micro plate reader to obtain values. A reference chart was used to calculate the concentration by comparing the optical density (OD) of the samples to the standard curves respectively.

Statistical Analysis

The software used was Statistical package for social sciences (SPSS) program-version 25 for data analysis. The Statistics analysis involved descriptive statistics to summarize the prevalence of genital mycoplasma species and other vaginal pathogens across different age groups and infection status, using counts (N), percentages (%), means and Standard deviations. The chi-square ($x^{2)}$ test of independence was applied to assess associations between categorical variables such as age, risk factors, and presence of pathogens, with statistical significant set as $p < 0.005$. Additionally, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of the culture method were calculated using PCR as the gold standard. These metrics evaluated the diagnostic performance of the culture method in detecting *M. hominis, and Ureaplasma species.* Furthermore, One-way analysis of variance (Anova) was used to compare mean CD4 counts and Cytokine levels (IL_1β and IL-10) across groups defined by pathogen or virulent gene presence.

RESULTS

Table 1 shows the levels of inflammatory cytokines IL-1Beta and IL-10 in patients with genital mycoplasmas and other vaginal pathogens. For IL-1Beta, patients with *Mycoplasma genitalium, Mycoplasma hominis, and Ureaplasma urealyticum* showed slightly higher cytokine levels compared to negative cases, though the

differences were not statistically significant ($p > 0.05$). Pathogens like *Mycoplasma pirium, Mycoplasma fermentans, and Mycoplasma penetrans* had no measurable IL-1Beta levels in positive cases, as indicated by 0.00 ± 0.00. Other pathogens, including *Staphylococcus aureus, E. coli, Klebsiella species, and Candida species*, displayed minimal differences in IL-1Beta levels between positive and negative cases, none of which reached statistical significance ($p > 0.05$). Interestingly, *Beta-hemolytic Streptococci* showed a higher mean IL-1Beta level in positive cases (18.36 ± 1.93) compared to negatives (16.14 ± 2.94), though the difference was not statistically significant ($p = 0.195$). For IL-10, positive cases of *Mycoplasma genitalium, Mycoplasma hominis, and Ureaplasma parvum Mycoplasma penetrans*exhibited higher levels compared to negative cases, though again, the differences were not significant ($p > 0.05$). Certain pathogens like *Mycoplasma pirium, Mycoplasma fermentans,* and showed no detectable IL-10 levels in positive cases. Conversely, pathogens like *Klebsiella and E. faecalis* showed lower IL-10 levels in positive cases compared to negatives, but the differences remained statistically insignificant. Interestingly, Candida presented a high variation in IL-10 levels among positive cases (512.66 ± 602.29) compared to negatives (422.36 ± 197.73), though this was also not statistically significant ($p = 0.104$).

Table 1. Level of inflammatory cytokines (IL-1Beta and IL-10) of patients with genital mycoplasmas and other vaginal pathogens

Genita mycoplasmaand other vaginal pathogens	IL-1Beta		IL-10	
	Positive	Negative	Positive	Negative
Mycoplasma genitalium	16.36±1.92	16.16±2.99	526.13±172.21	426.52±271.50
F(p-value)	1.536(0.813)		0.033(0.192)	
Mycoplasma hominis	16.38±2.39	16.05±3.25	469.11±374.04	407.15±162.48
F(p-value)	0.357(0.438)		5.826(0.072)	
Ureaplasma urealyticum	17.34±2.94	16.12±2.93	450.67±266.59	430.52±268.56
F(p-value)	0.173(0.141)		1.436(0.785)	
Mycoplasma pirium	0.00±0.00	16.17±2.94	0.00±0.00	431.64±267.85
F(p-value)	-		-	
Mycoplasma fermentas	0.00±0.00	16.17±2.94	0.00±0.00	431.64±267.85
F(p-value)	-		-	
Ureaplasma parvum	15.49±0.80	16.19±2.97	521.11±167.76	429.09±270.06
F(p-value)	3.788(0.537)		0.050(0.371)	
Mycoplasma penetrans	0.00±0.00	16.17±2.94	0.00±0.00	431.64±267.85
F(p-value)	-		-	
Staphylococcus aureus	16.05±2.76	16.18±2.96	434.13±150.04	431.38±277.66
F(p-value)	0.224(0.831)		0.640(0.962)	
E.coli	16.09±2.64	16.18±2.96	388.31±172.11	434.18.272.57
F(p-value)	0.048(0.914)		0.001(0.535)	
Klebsiella pneumonia	15.18±2.86	16.21±2.94	283.87±99.20	437.72±271.05
F(p-value)	0.350(0.277)		0.478(0.075)	
E. *Faecalis*	15.66±2.56	16.20±2.96	382.63±208.58	434.72±271.32
F(p-value)	0.004(0.504)		0.159(0.466)	
Candida specie	16.06±2.84	16.18±2.95	512.66±602.29	422.36±197.73
F(p-value)	0.724(0.845)		7.036(0.104)	
Alpha Hemolytic Streptococcus	14.57±2.02	16.20±2.95	395.09±55.68	432.38±270.52
F(p-value)	0.445(0.220)		0.732(0.759)	
Beta. Hemolytic Streptococcus	18.36±1.93	16.14±2.94	352.41±108.53	432.59±269.26
F(p-value)	0.313(0.195)		0.200(0.607)	
T.vaginalis	16.04±2.98	16.18±2.98	389.99±114.94	433.53±272.86
F(p-value)	1.205(0.882)		0.438(0.599)	

DISCUSSION

The present study revealed no statistically significant relationship between the presence of *Mycoplasma hominis* virulence genes (P120 and VAA) and the systemic levels of the cytokines IL-1Beta and IL-10, suggesting that these specific surface proteins may not be the primary drivers of the differential cytokine response observed. A lack of association between P120/VAA genotype and systemic IL-1β or IL-10 does not negate pathogenic relevance of these proteins but

reframes their likely role in host interaction. Because P120 and VAA are major loci of antigenic variation and adhesion, their primary contribution may be to persistence, immune escape, and tissue tropism rather than to driving systemic cytokine profiles [13]. The analysis of inflammatory cytokine levels in patients with genital mycoplasmas and other vaginal pathogens revealed trends that, although not statistically significant ($p > 0.05$), suggest a complex immune response. Patients with *Mycoplasma genitalium*, *Mycoplasma hominis,* and *Ureaplasma urealyticum* showed slightly higher IL-1Beta levels. This finding is consistent with the study by Dehon et al. [14], which reported significantly higher levels of interleukin 1β (IL-1β) in cervical secretions from HIV-infected women with chronic *Mycoplasma genitalium* infection. However, whereas Dehon et al.[14] found significant associations specifically for *M. genitalium*, our study observed similar trends for *M. genitalium, M. hominis*, and *U.urealyticum*, albeit without statistical significance, highlighting the need for further investigation into the immune responses elicited by these pathogens. Additionally, while most of the other pathogens (Staphylococcus, *E. coli*, Klebsiella, and Candida) showed minimal differences in IL-1Beta levels between positive and negative cases, Beta-hemolytic streptococci exhibited a notable increase in mean IL-1Beta levels in positive cases compared to negatives (18.36 ± 1.93 vs 16.14 ± 2.94). Although the difference was not statistically significant ($p = 0.195$), the direction suggests that Beta-hemolytic streptococci may induce a more pronounced inflammatory response compared to the other pathogens listed. Beta-hemolytic Streptococci, such as GAS (Group A streptococcus), are known to elicit a robust inflammatory response characterized by IL-1β activation, which is crucial for protective immunity, contrasting with non-mycoplasma pathogens that may not induce such a pronounced response [15].

The absence of detectable IL-10 levels in positive cases of *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum* and *Ureaplasma parvum* may suggest that these pathogens either suppress or do not induce a significant anti-inflammatory response, as mediated by IL-10. This could imply that the immune response to these pathogens is skewed towards a pro-inflammatory profile, potentially contributing to tissue damage or disease pathogenesis [16]. Interestingly, Candida presented a high variation in IL-10 levels among positive cases (512.66 ± 602.29) compared to negatives (422.36 ± 197.73). Although the difference in IL-10 levels was not statistically significant ($p = 0.104$), the higher mean and variability in Candida-positive cases may reflect individual differences in immune response. This trend aligns with evidence that IL-10 plays a modulatory role in fungal infections and may vary with disease severity or host factors [17].

CONCLUSION

The findings indicate that **genital *Mycoplasma*, *Ureaplasma*, and other vaginal pathogens were not significantly associated with circulating IL-1β or IL-10 levels** in the study population. Although some pathogen-positive groups, particularly those with *Mycoplasma genitalium*, *Ureaplasma parvum*, and *Candida* species, demonstrated a tendency toward higher IL-10 concentrations, these differences were not statistically significant. Similarly, IL-1β levels showed no significant differences between pathogen-positive and pathogen-negative participants. Overall, the results suggest that detection of these genital pathogens does not necessarily correspond to measurable systemic alterations in the inflammatory cytokine profile. Further studies incorporating local genital cytokine measurements, pathogen load, clinical symptoms, and longitudinal follow-up are warranted to better characterize the immunological response to genital tract infections.

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